Biofilm Matrixome: Extracellular Components in Structured Microbial Communities

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Abstract: Biofilms consist of microbial communities embedded in a 3D extracellular matrix. The matrix is composed of a complex array of extracellular polymeric substances (EPS) that contribute to the unique attributes of biofilm lifestyle and virulence. This ensemble of chemically and functionally diverse biomolecules is termed the 'matrixome'. The composition and mechanisms of EPS matrix formation, and its role in biofilm biology, function, and microenvironment are being revealed. This perspective article highlights recent advances about the multifaceted role of the 'matrixome' in the development, physical-chemical properties, and virulence of biofilms. We emphasize that targeting biofilm-specific conditions such as the matrixome could lead to precise and effective antibiofilm approaches. We also discuss the limited knowledge in the context of polymicrobial biofilms, and the need for more in-depth analyses of the EPS matrix in mixed communities that are associated with many human infectious diseases. Keywords: extracellular matrix; extracellular polymeric substances (EPSs); microenvironments; polymicrobial biofilm; spatial organization; virulence.

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Review

Biofilm Matrixome: Extracellular Components in Structured Microbial Communities

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Biofilms consist of microbial communities embedded in a 3D extracellular matrix. The matrix is composed of a complex array of extracellular polymeric substances (EPS) that contribute to the unique attributes of biofilm lifestyle and virulence. This ensemble of chemically and functionally diverse biomolecules is termed the 'matrixome'. The composition and mechanisms of EPS matrix formation, and its role in biofilm biology, function, and microenvironment are being revealed. This perspective article highlights recent advances about the multifaceted role of the 'matrixome' in the development, physical–chemical properties, and virulence of biofilms. We emphasize that targeting biofilm-specific conditions such as the matrixome could lead to precise and effective antibiofilm approaches. We also discuss the limited knowledge in the context of polymicrobial biofilms, and the need for more in-depth analyses of the EPS matrix in mixed communities that are associated with many human infectious diseases.

Highlights

The 'matrixome' is the inventory of currently known biomolecules (polysaccharides, nucleic acids, proteins, lipids, and lipoproteins) and their molecular, structural, and functional diversity associated with biofilm assembly, and its physicochemical and virulence attributes.

The structural and biochemical properties of the matrixome provide the emergent properties of biofilms, including surface adhesion, spatial and chemical heterogeneities, synergistic/competitive polymicrobial interactions, antimicrobial recalcitrance, and biofilm virulence.

Combinatorial treatment strategies are crucial to eradicate biofilms by targeting the functionally and structurally complex extracellular polymeric matrix and embedded microbial cells.

Due to limited knowledge of the polymicrobial EPS matrix there is an urgent need for more experimental polymicrobial biofilm and *in vivo* mechanistic studies.

Introduction

Biofilm is defined as a structured community of microbial cells firmly attached to a surface and embedded in a matrix composed of **extracellular polymeric substances** (**EPS**) (see Glossary). The EPS consist of exopolysaccharides, nucleic acids (**eDNA** and eRNA [1]), proteins, lipids, and other biomolecules. The formation of sessile biofilm communities involves highly complex and dynamic events whereby EPS play key structural and functional roles that are essential for the **emergent properties of biofilms** [1]. EPS promote microbial adhesion to biotic and abiotic surfaces. Once attached, further EPS production forms a matrix that surrounds and cements cells together, keeping them in close proximity and allowing intercellular interactions in a confined space [1]. The EPS matrix also provides mechanically stable and complex chemical microenvironments that are fundamental for the biofilm lifestyle [2]. Besides offering structural stability and a functional environment, EPS also enhance biofilm tolerance to antimicrobials and immune cells [3,4]. In this review we highlight the composition and the diverse functions of biofilm EPS matrices, collectively termed the 'matrixome', and their role in virulence from model organisms. We also bring these findings into perspective in the context of polymicrobial communities often found in biofilms associated with infectious diseases. We hope that this article will stimulate new questions, hypotheses, and approaches that may lead to a better understanding of the biofilm matrix and its role in biofilm-associated infections.

The Matrixome: The Composition and Functional Diversity of the EPS Matrix

Over the years, the functional role of EPS has been increasingly recognized and intimately linked with the emergent properties of biofilms [1,5]. Biofilm studies in the field of dental medicine provide an excellent source of information about the composition and functional roles of the EPS matrix (Box 1). Here we use the term 'matrixome', adapted from 'matrisome' used traditionally in the field of eukaryotic cell biology (see Box 2), to define the entire inventory of currently known biomolecules, and their molecular, structural, and functional diversity, associated with biofilm assembly

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Box 1. The Dental Biofilm: Clues from Ancient to Modern Times

One of the first descriptions of bacteria living in biofilms can be traced back to a letter written by Antonie van Leeuwenhoek to the Royal Society of London in 1683 describing a vast number of microorganisms in the dental plaque of his own teeth. Since then biofilms have been extensively studied in different disciplines in both biomedical and industrial fields. Studies with 'dental plaque' have been at the forefront of microbiome and EPS analyses [91] and even in paleomicrobiology, serving as a source of information on the composition of ancient microbial and host biomolecules, including dietary components [92]. Many clues about ecology and dynamic changes of the human microbiota were derived from oral microbiome studies. Likewise, early work from Bernie Guggenheim and Bill Bowen in the 1960s and 1970s [93,94] revealed the first insights about the presence of abundant exopolysaccharides in the plaque associated with tooth decay, which have been confirmed using modern microscopy and spectroscopy techniques. Furthermore, the development of confocal laser scanning microscopy (CLSM) and fluorescence *in situ* hybridization (FISH) for matrix analysis, combined with *in vitro* multispecies systems, animal and human-based intraoral models [95,96], allowed in-depth investigations on the microbial and EPS matrix complexity revealing new insights into the 3D structure of biofilms. Indeed, some of the first images showing the diversity of EPS and microbiota structure and spatial organization were acquired from oral biofilms. The field of dental biofilm research will continually provide important information about the composition, structural organization, and function of EPS and the microbiome, which may be applicable to other polymicrobial infections and environmental communities.

Glossary

Biofilm virulence: intrinsic 'malevolence' of a biofilm, which is the collective attributes and factors that lead to infection of the host at the various attachment sites on both biotic and abiotic surfaces to cause disease onset and worsen disease severity.

eDNA: extracellular DNA that can be either a result of cell lysis or actively secreted; eDNA may interact with different EPS components contributing to the biofilm structural organization, serving as a nutrient source, while promoting protection against antimicrobials and horizontal gene transfer.

Emergent properties of biofilms: novel structures, activities, and patterns of a biofilm that are not identifiable or predictable through the study of planktonic bacterial cells. Emergent properties of biofilms include surface adhesion-cohesion, spatial organization, physical and social interactions, chemical heterogeneity, and increased tolerance to antimicrobials.

EPS degradation: therapeutic strategies that use exogenous EPS-degrading enzymes or other agents to disassemble the biofilm matrix. EPS degradation can weaken the biofilm structure to facilitate mechanical removal and enhance the killing efficacy of antimicrobials when applied through an adjunctive approach.

Extracellular polymeric substances (EPS): EPS can contain exopolysaccharides, fibrous and globular proteins (including extracellular enzymes), lipids, and nucleic acids (eDNA). These components form a matrix that can be surface-associated, secreted locally, or deposited on abiotic and biotic surfaces. The EPS matrix functions as a 'multifunctional scaffold' that supports and protects embedded bacteria, creates a heterogeneous chemical and physical milieu, while also serving as a nutrient source for resident microbes.

and its physicochemical and virulence attributes [2]. The composition and structure of EPS can vary greatly depending on the type of microorganisms, local shear stress, availability of nutrients/substrates, and the host environment [1]. EPS synthesis and spatial organization also differ between monospecies and multispecies microbial communities [5]. Thus far, a diverse array of biomolecules has been identified (Table 1). These can be grouped into two major categories: (i) associated with the cell surface, and (ii) secreted extracellularly (Figure 1). Some examples include cell-associated appendages such as flagella, type IV pili, and functional amyloids that modulate bacterial adhesion, mechanical stability, and autoimmune responses. Conversely, secreted bacterial exopolysaccharides, proteins, eDNA, and eRNA released extracellularly contribute with matrix scaffolding and function [1,5]. Although we focus on microbially produced EPS, biomolecules acquired from the host or surrounding environment should be included as part of the matrixome. Host proteins and glycoproteins (e.g., the salivary proteins) were found to contribute to the matrix scaffold and assist microbial attachment while serving as a microbial nutrient source [6]. The EPS components have been shown to be vital for the structural and functional attributes of the biofilm, which can be generally divided into physical and chemical properties [3].

Minerals are the result of biomineralization processes tightly regulated by the environment or bacteria and serve as an essential component of the EPS [7,8]. An earlier report showed that minerals structurally support the morphogenesis of bacterial colonies in the Gram-positive bacterium *Bacillus subtilis* [9] and in *Mycobacterium* species [8,9]. Biogenic minerals also provide structural integrity to biofilm matrix and act as a scaffold to protect bacterial cells from shear forces and antimicrobial agents [10]. An earlier report demonstrated the presence of $CaCO_3$ in the matrix of *B. subtilis* and *Mycobacterium smegmatis* biofilms, and implied the association of matrix mineral with other bacterial species [9]. Another mineral, namely calcite, has been linked to various medical conditions. In particular, biofilms of *Proteus mirabilis*, *Proteus vulgaris*, and *Providencia rettgeri* cause extensive catheter encrustation by calcium and magnesium minerals [7], while the Gram-negative pathogen *Pseudomonas aeruginosa* generates calcite during biofilm formation [11].

Physical Properties Provided by EPS

Adhesion–cohesion, scaffolding, mechanical stability, and protection belong to the most prominent EPS functions. Collective properties of biofilms, such as collective migration and spreading, result from cell–cell interactions and allow for an effective adaptation of bacterial masses to adverse environmental conditions. The role of EPS in collective biofilm properties has been

Table 1. Composition and Functions of Extracellular Polymeric Substances (EPS) in Biofilms from Some Model Organisms

Class	Microorganism[a]	Name	Location	Function
Polysaccharides	*Bacillus subtilis*	*epsA-epsO* operon-encoded exopolysaccharide	Extracellular	Adhesion, scaffolding, stability
		γ-PGA (poly-γ-glutamate)	Extracellular	Adhesion, scaffolding, sorption, nutrient
	Staphylococcus aureus	Polysaccharide intercellular adhesin (PIA) or poly-β (1-6)-*N*-acetylglucosamine (PNAG)	Extracellular	Adhesion, cohesion, scaffolding, stability, protection against antibiotics
	Streptococcus mutans	Glucans/fructans	Extracellular/cell-associated	Adhesion, cohesion, scaffolding, stability, cell-to-cell binding, acidic microenvironment, protection against antimicrobials, nutrient
	Pseudomonas aeruginosa	Psl	Extracellular/cell-associated	Adhesion, scaffolding, stability, protection against immune response, cell-to-cell binding
		Pel	Extracellular/cell-associated	Adhesion, scaffolding, stability, cell-to-cell binding, protection against antibiotics
		Alginate	Extracellular	Adhesion, scaffolding, water/nutrient retention, protection against harsh environments/immune response/antimicrobials, stability
	Vibrio cholerae	*Vibrio* polysaccharide (VPS)	Extracellular/cell-associated	Adhesion, cohesion, scaffolding, stability
	Candida albicans	α-mannans	Extracellular Cell wall	Forming mannan-glucan complex (MGCx), scaffolding, protection, antifungal resistance, bacterial–fungal interaction
		β-glucans	Extracellular Cell wall	Forming mannan-glucan complex (MGCx), scaffolding, protection, antifungal resistance, bacterial–fungal interaction
Proteins	*Bacillus subtilis*	Biofilm surface layer protein (BslA)	Extracellular	Surface hydrophobicity, protection
		Translocation-dependent antimicrobial spore component (TasA)/TasA anchoring and assembly protein (TapA)	Extracellular Cell wall	Scaffolding, cell-to-cell binding
		Flagellum	Cell-associated	Adhesion, motility, mechanosensing
	Staphylococcus aureus	Fibronectin-binding proteins (FnBPs)	Cell-associated/extracellular	Adhesion, cell-to-cell binding
		Staphylococcal Protein A (SpA)	Cell-associated/extracellular	Adhesion, cell-to-cell binding, immune evasion
		S. aureus surface protein G (SasG)	Cell-associated/extracellular	Adhesion, cell-to-cell binding
		Biofilm associated protein (BAP)	Extracellular	Adhesion, cell-to-cell binding, scaffolding, stability
		Phenol-soluble modulins (PSMs)	Extracellular	Proinflammatory, lysing of host cells, biofilm spreading, scaffolding
	Streptococcus mutans	Glucosyltransferases (Gtf)/ fructosyltransferases (Ftf)	Cell-associated/extracellular	EPS production
		Dextranase	Extracellular	EPS degradation/remodeling
		P1 (also referred to as Antigen I/II)	Cell-associated	Adhesion, cell-to-cell binding
		Glucan binding proteins (GbpA, GbpB, GbpC)	Cell-associated /extracellular	Adhesion, cohesion, stability
	Pseudomonas aeruginosa	Type IV pilins (T4P)	Cell-associated	Adhesion, scaffolding, twitching motility, mechanosensing
		Lectins (LecA/LecB)	Cell-associated/extracellular	Adhesion, cell-to-cell binding, stability, cytotoxin

Table 1. (continued)

Class	Microorganism[a]	Name	Location	Function
	Vibrio cholerae	Biofilm-associated protein (Bap1)	Cell-associated/extracellular	Adhesion, scaffolding, hydrophobicity, stability, protection
		Rugosity and biofilm modulators (RbmA/RbmC)	Cell-associated/ extracellular	RbmA: cell-to-cell binding RbmC: scaffolding, stability
		Mannose-sensitive hemagglutinin (MSHA) pili	Cell-associated	Adhesion, motility, mechanosensing
		Flagellum	Cell-associated	Adhesion, motility, mechanosensing
	Candida albicans	Agglutinin-like sequence protein (Als)	Cell wall	Adhesion, cell-to-cell binding, bacterial-fungal interaction
		Hyphal wall proteins (Hwp)	Cell wall	Adhesion, cell-to-cell binding
		Heat-shock proteins (Hsp70)	Cell-associated/ extracellular	Unknown function in EPS
		Functionally classified enzymes	Extracellular	Metabolism of carbohydrate/amino acid/lipid/nucleotide/energy/vitamins, translation, transport, catabolism, folding, sorting, replication, and repair
Nucleic acids	Wide distribution in bacteria, archaea, and fungi	eDNA	Extracellular	Scaffolding, adhesion, cohesion, nutrient source, DNA damage repair, gene transfer, interaction with other matrix components
Lipids	*Candida albicans*	Glycerolipids, sphingolipids	Cell-wall/extracellular	Unknown
	Staphylococcus aureus	Teichoic and lipoteichoic acids	Cell-associated/extracellular	Adhesion, cohesion, protection, immune evasion
Lipopolysaccharides	Wide distribution in Gram-negative bacteria	LPS (endotoxin)	Cell-associated/extracellular	Adhesion, colonization and host invasion, activation of immune response

[a]Additional details about the EPS components, including from other microbes (such as *Escherichia coli*) can be found in the following references [3,5,16,18–20,97–100].

highlighted in previous studies [12–15]. The EPS can promote cell adhesion to solid substrates and cohesion among bacterial cells, eventually leading to the development of structured cell clusters, often termed microcolonies [5]. Most of the current knowledge has been generated using biofilm-forming model organisms, particularly *B. subtilis, Candida albicans, Escherichia coli*, *Staphylococcus aureus, Streptococcus mutans, P. aeruginosa,* and *Vibrio cholerae*. Excellent in-depth reviews discuss in detail the composition and functional role of EPS within various matrices produced by these organisms as single-species biofilms [1,3,5,16–18]. Here, we focus on how different organisms employ unique approaches using EPS, while bringing them into the context of mixed or polymicrobial settings whenever details are available.

The initial bacterial adhesion often involves the classic adhesin–receptor interactions (Table 1) but also intriguing surface-scanning and sensing mechanisms, as recently shown in *V. cholerae* and *B. subtilis*, whereby extracellular appendages such as flagella act as mechanosensors [1,19,20]. Greater details on the EPS-mediated adhesion have been elucidated in *P. aeruginosa, S. mutans, B. subtilis*, and *V. cholerae* at the single-cell level with single polymer precision [21–29]. Moreover, single-cell live imaging of *V. cholerae* biofilms revealed that the adhesion-relevant EPS protein Bap1, secreted by the founder cells, remains substratum-bound in mature biofilms [24]. Another *V. cholerae* matrix protein (RbmA) has been recently shown to exhibit adhesin-like properties mediating surface attachment [28]. Intriguingly, initial *S. mutans* colonizers appear to sense adhesion

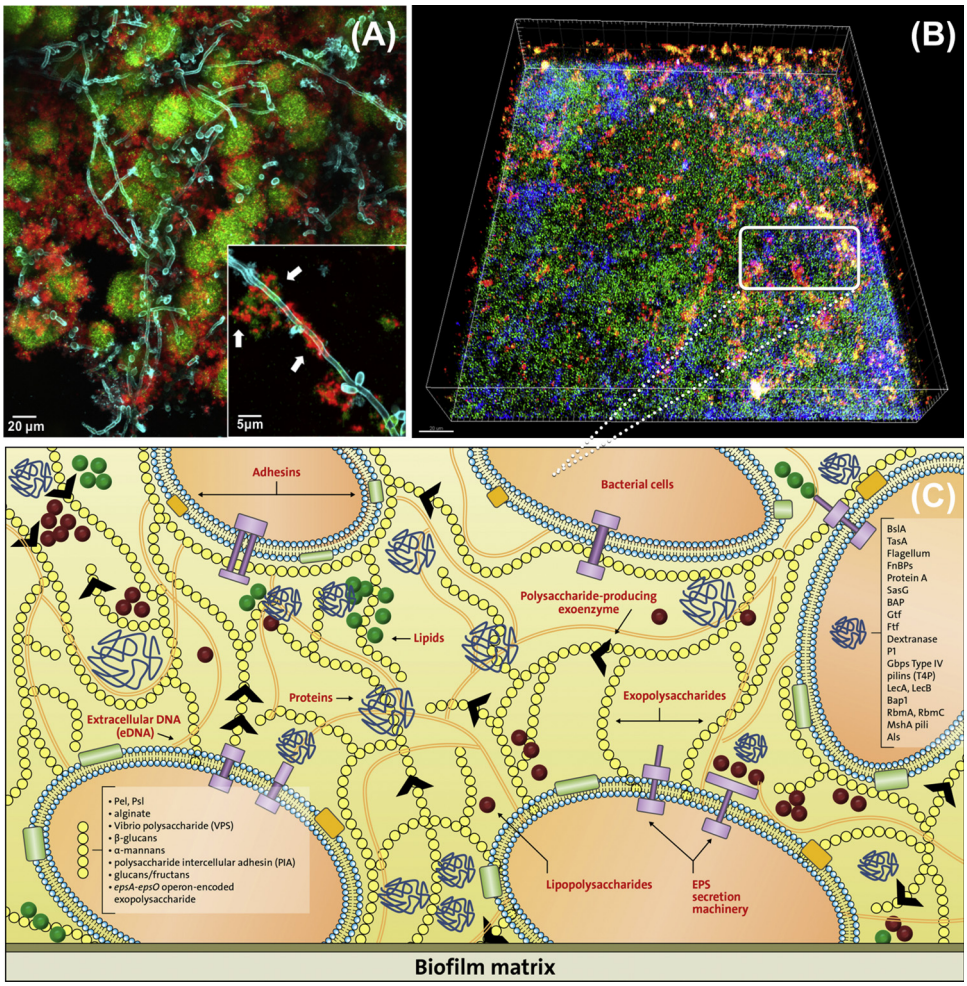

Figure 1. Composition and Functions of Biofilm Matrix in Structured Microbial Communities. Panel A depicts confocal fluorescence images of developed cross-kingdom dental biofilms within extracellular matrix (ECM) (red); inset shows *Streptococcus mutans* (green)–*Candida albicans* (cyan) interactions mediated by ECM (white arrows). Panel B depicts 3D reconstructions of *in vitro* oral biofilms after matrix staining and confocal laser scanning microscopy (CLSM). Specifically, six-species biofilms consisting of *Streptococcus mutans*, *Streptococcus oralis*, *Actinomyces oris*, *Fusobacterium nucleatum*, *Veillonella dispar*, and *Candida albicans* were grown anaerobically on pellicle-coated hydroxyapatite disks. The visualization of the treated biofilms was aided by matrix staining and CLSM using anti-DNA antibodies, streptavidin (Cy3), calcofluor, SYPRO Ruby, and YoPro-1/Sytox. Green, bacteria; blue, exopolysaccharides; red, extracellular DNA (eDNA); yellow, proteins. Panel C is a schematic representation of the main components of the biofilm matrix and their functions. The biofilm matrix consists of a wide array of functional biomolecules such as exopolysaccharides (Pel, Psl, alginate, *Vibrio* polysaccharide (VPS), β-glucans, α-mannans, polysaccharide intercellular adhesin (PIA), glucans/fructans, epsA-epsO, operon-encoded exopolysaccharide), proteins (BslA, TasA, Flagellum, FnBPs, Protein A, SasG, BAP, Gtf, Ftf, Dextranase, P1, Gbps, Type IV pilins (T4P), LecA ,LecB, Bap1, RbmA, RbmC, MshA pili, Als) nucleic acids, and lipids that organize into an extracellular matrix. This matrix serves as a scaffold for structural support and a dynamic milieu that provides varying chemical and physical signals to microbial communities, promoting a biofilm lifestyle.

forces that externally trigger the emergent biofilm properties over a specific distance above a substratum surface through quorum sensing [29].

EPS also promotes cell–cell cohesion (including interspecies recognition) to facilitate microbial aggregation and biofilm formation [1]. Other interspecies interactions depend on mechanosensors or

Box 2. Dynamic Cell–Matrix Interactions in 3D Tissue Microenvironments

Extensive research in cell and developmental biology established that eukaryotic cells sense both physical and chemical cues in their extracellular matrix (termed ECM), which triggers cellular responses that regulate cellular functions, including remodeling their surrounding 3D matrix. This reciprocal, bidirectional, and highly dynamic interaction between cells and matrix affects all facets of cell biology and pathology by modulating tissue and organ morphogenesis, homeostasis, and tumorigenesis. Local eukaryotic tissue microenvironments differ substantially in molecular composition, density, porosity, stiffness, and other features. Similar dynamic cell–matrix interactions and microenvironment heterogeneity occur in biofilms, where microbial cells such as bacteria and fungi adhere and generate a surrounding EPS matrix. Thus, relevant concepts concerning eukaryotic cell–matrix interactions, despite some fundamental differences, could be applied to enhance understanding of the biofilm developmental biology and expression of virulence. The eukaryotic ECM has well characterized functions as a chemical reservoir of growth factors, cytokines, and proteases, and the ECM can limit the diffusive range, accessibility, and concentrations of signaling ligands, providing spatial, chemical, and mechanical cues to activate intracellular signaling cascades. For example, matrix glycoproteins and proteoglycans can enhance signaling and adhesive functions, while growth factors and cytokines incorporated within ECM can locally stimulate adherent cells. Conversely, the mechanical characteristics of ECM, such as rigidity, porosity, and cross-linking, can be sensed by eukaryotic cells through mechanotransduction, affecting proliferation and gene expression locally. Matrix stiffness can alter differentiation, signaling, and induce tumorigenesis. Within tumors, there are often local regions of hypoxia, acidic pH, and the release of proteases and other cellular constituents. During tumor metastasis, cancer cells can invade or disperse to distant sites through complex processes of local matrix or target-tissue microenvironmental remodeling. Conceptually, similar processes appear to exist during biofilm development and dispersal. The biofilm EPS matrix provides highly structured yet spatially and chemically heterogeneous environments that locally affect cellular physiology, transcriptional activity, and survival that, albeit described, still needs further mechanistic elucidation. Advances in eukaryotic matrix biology and cell–matrix interactions can help to stimulate new questions and insights to the field of biofilm biology, which in turn may also provide new approaches for tissue engineering and cancer biology.

specific adhesin (protein)–receptor (saccharide) pairs. In general, the EPS-based matrix mediates biofilm assembly as follows. (i) The EPS formed at the site of adhesion (produced on bacterial surfaces or secreted on the surface of attachment) form an initial polymeric matrix promoting microbial colonization and cell clustering. (ii) Continuous EPS production *in situ* further expands the matrix three-dimensionally while forming a core of EPS-enmeshed bacterial cells. (iii) This core provides a supporting framework, facilitating the development of 3D clusters and aggregates (or microcolonies). The transition from initial cell clustering to microcolony appears to be conserved among different biofilm-forming model organisms, such as *P. aeruginosa, B. subtilis*, *V. cholerae*, and *S. mutans* [3,16,17,21,24–29]. For instance, *S. mutans* relies on EPS-producing exoenzymes, termed glucosyltransferases (Gtfs), which synthesize glucans *in situ* using host diet sugars as substrates [5,30]. Interestingly, Gtfs bind on the surface of many oral microbes (e.g., *Actinomyces viscosus, Lactobacillus casei*, and *C. albicans*), even those that do not synthesize Gtfs [5], promoting interspecies and interkingdom coadhesion [31]. Recently, a key role for EPS-protein TasA, produced by *B. subtilis*, in mediating interspecies aggregation with streptococci was highlighted [32]. The structural importance of EPS in ecological interactions in mixed communities was shown using an individual-based multispecies biofilm model in which EPS-producing microbes that build aggregates have a competitive advantage over the non-EPS-producing microbes during initial adhesion [33].

The EPS production *in situ* leads to the formation of complex polymeric 3D matrix scaffolds incorporating a plethora of densely packed microbes and providing cohesion, mechanical stability in the resulting highly compartmentalized biofilms [16]. Recent reports elucidated the scaffolding role of highly stable networks of amyloid-insoluble aggregates comprising an assembly of protein monomers in a variety of bacterial biofilms (as reviewed elsewhere [34]). For example, the nonattached P1 adhesin (AgI/II) of *S. mutans* [35], the nonamyloidogenic TasA formed by *Bacillus* sp. [36], the Fap fibers in *Pseudomonas* sp. [37], and the Bap fibers of *S. aureus* [38] were found to form functional amyloids (amyloid-like fibers) enhancing the structural stability of biofilms and providing protection for the bacteria. Recently, structured illumination super-resolution microscopy also revealed coordinated explosive cell lysis of a specific subpopulation of *P. aeruginosa* that releases eDNA and other polymers to help build-up the matrix [39], while confocal fluorescence microscopy

enabled the visualization of specific colocalized EPS components and interactions between Pel and eDNA within *P. aeruginosa* biofilms [40]. However, the contribution of individual matrix components to emergent physical properties of multispecies-species biofilms remains poorly understood.

As biofilm becomes established, an essential physical feature of EPS is to provide physical stability and resistance to mechanical removal, antimicrobials, and host immunity. EPS-associated viscoelasticity of mature biofilms (afforded by exopolysaccharides and eDNA) makes their detachment from the substratum challenging even under sustained fluid shear stress or high mechanical pressure [17]. Interestingly, increased EPS production also reflects a highly adaptive/protective response of biofilms to environmental stress factors such as high shear as revealed by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy combined with tribometry [41].

In addition to mechanical resistance, EPS also promote protection against antimicrobials and enhanced drug tolerance [1,16–18,35,38–40]. EPS can act as a diffusion-limiting barrier against various antimicrobials resulting in limited drug access into the deeper layers of the biofilm [42]. Retarded penetration due to the reaction of antimicrobials with EPS components, (e.g., positively charged agents likely bind to negatively charged polymers) also contributes to the antimicrobial tolerance of biofilms [4], enabling inactivation or degradation of antimicrobials by enzymes present in biofilm matrix [43]. Interaction of antimicrobials with biofilm matrix can also alter specific genetic determinants of antimicrobial tolerance. Recently, an intriguing interkingdom EPS-mediated antimicrobial resistance was found in mixed-species biofilms containing *C. albicans* and bacteria. Exopolysaccharides (β-1,3-glucan cell-wall component) secreted by *C. albicans* prevented penetration of antibacterial drugs (such as vancomycin) providing enhanced antimicrobial protection for *S. aureus* within mixed biofilms [44,45]. Conversely, *S. mutans* glucans surrounding *Candida* cells directly bound and sequestered an antifungal agent (fluconazole), reducing drug uptake and enhancing *C. albicans* tolerance within mixed biofilms [46]. Similarly, a three-species (*P. aeruginosa*, *S. aureus*, and *C. albicans*) interkingdom biofilm yielded resistance to flucloxacillin, ciprofloxacin, or fluconazole. This can be presumably attributed to the diffusion-limiting effects of EPS-matrix or the presence of EPS-associated degradative enzymes [47,48]. Yet, the exact mechanisms of the EPS-mediated resistance of biofilms to antibiotics have not been elucidated. Another example is the biofilm formed by *Bacillus* sp. which presents as a highly hydrophobic community that resists wetting by water, solvents, and biocides by forming a hydrophobic film that coats the biofilm surface and renders it water-repellent. This remarkable property is conferred by the secreted BslA and orthologous proteins that can self-assemble into an organized lattice at the water–air interface [49,50].

However, it is important to keep in mind that biofilm formation may be essential for some beneficial functions. *B. subtilis*, for example, has gained interest for its probiotic properties as it can effectively maintain a favorable balance of microflora in the gastrointestinal tract. In order to survive processing and storage of food, as well as passage through the upper gastrointestinal tract, *B. subtilis* produces an extracellular matrix that protects it from stressful environments [51]. In addition, *B. subtilis* can effectively protect plants against diverse microbial threats. The protein matrix component TasA and the exopolysaccharide have both been shown to be essential for effective plant-root colonization in both *Arabidopsis* and tomato plants [16].

Chemical Properties Associated with EPS Such compartmentalized architecture confers cohesive yet highly heterogeneous environments within a 3D matrix scaffold. EPS as a physical boundary can sequester or trap diverse substances while also influencing the diffusion of various molecules inside the biofilm, thereby creating nutritional and chemical gradients, including oxygen, pH, signaling molecules, inorganic ions, metabolites, and other solutes, across the biofilm 3D architecture. As a result, heterogeneous biofilm microenvironments with varying chemical

milieus ensue, which can be further altered by the interactions between EPS and local microbial metabolism [52,53]. Localized oxygen heterogeneity within *B. subtilis* biofilms modulated the function of the matrix protein BslA by inducing redox-dependent bifunctionality. Specifically, BslA presented as a dimer on the exposed oxygen-rich biofilm surface, whereas in deeper anoxic biofilm layers BslA dimerization was inhibited, triggering nutrient uptake [49]. Further studies on *P. aeruginosa* and *E. coli* biofilms underlined the differential EPS-related gene expression and metabolic specialization between microbial subpopulations with varied oxygen abundance [54,55]. For instance, in the absence of the redox-dependent cytochrome *bd*-expressing subpopulation (responsible for aerobic respiration under hypoxic conditions) in *E. coli* biofilms during acute urinary tract infections, substantially less EPS was produced [55]. Extracellular pH microenvironments have been spatially assessed using noninvasive *in situ* pH measurement via multiphoton confocal microscopy showing a highly heterogeneous pH distribution across intact 3D biofilm architecture [56–58]. At a single-microcolony level, the EPS matrix allowed a gradient of acidic microenvironments to form that directly regulated the differential expression of pH-responsive *atp*B by *S. mutans* distributed across various locations throughout the biofilm structure [56].

Another prominent chemical function of the biofilm matrix is its role as local nutrient reservoir of various biomolecules, for example, fermentable polysaccharides [2,16,59]. A recent report revealed that due to osmotic pressure differences in *V. cholerae* biofilms, the microbial colonies physically swell, thereby maximizing their contact with nutritious surfaces and thus, nutrient uptake [60]. Consistent with this finding, nutrient availability in dual-species (*C. albicans*/*Streptococcus oralis*) biofilms seems to promote biofilm expansion without interfering with its mechanical stability [61]. Notably, the biofilm matrix can also act as an external digestion system due to the immobilization of exoenzymes which actively participate in EPS remodeling (synthesis and degradation) and metabolism of diverse substrates [5]. In *S. mutans* biofilms, dextranase and fructanase directly degrade EPS components, for example soluble glucans and fructans, respectively, allowing for the production of fermentable polysaccharides on-site that can be utilized during starvation [5]. The eDNA is considered as a carbon source that can influence biofilm dispersal [62]. Interestingly, anionic EPS components and eDNA can serve as cation chelators and even generate a cation-deficient environment, thereby promoting antimicrobial resistance [63]. However, the role of other matrix components in nutrient deposition and the formation of chemical gradients over time needs further elucidation, especially in polymicrobial biofilms.

Biofilms contain a variety of heterogenous, EPS-delineated milieus able to alter local gene expression, metabolic activity, and importantly intercellular signaling between different species within the biofilm mass [2,3,64–66]. Recent analyses of pathways modulating biofilm matrix gene expression have revealed a plethora of extra- and intracellular signaling molecules [67]. Quorum sensing (QS) is highly associated with matrix, thereby affecting biofilm functions. In *P. aeruginosa* biofilms, exopolysaccharides facilitate the absorption of QS molecules within the biofilm, whereas biofilm matrix can either activate or deactivate QS processes [68]. Nevertheless, the detailed pathways by which matrix regulates chemical or mechanical sensing/signaling remain unknown for both single- and multispecies biofilms.

The Matrixome: Amplifying the Biofilm Virulence

The physical and chemical properties of the biofilm matrix provide emergent properties of the biofilms that are critical for biofilm existence and expression of virulence. These include adhesion–cohesion, pathological microenvironments, mechanical and drug resistance that can lead to disease onset and worsen disease severity. Here, we focus on host infection animal models reporting the potential role of EPS in **biofilm virulence**. However, we realize that this is a nascent field in polymicrobial settings and that there is a very limited number of studies investigating *in vivo.*

Matrix-Associated Biofilm Virulence

The importance of the matrix in collective microbial behavior and function, as well as for tolerance of antimicrobials, is being increasingly recognized and considered integral to biofilm virulence [1,3–5,16–18,69]. The matrix plays an essential role in the pathogenesis of human and animal diseases as several matrix constituents are recognized as potential virulence factors [1–5,16,17,70]. Staphylococcal EPS – for example, eDNA and poly-*N*-acetylglucosamine surface polysaccharide (PNAG) – mediate numerous virulence traits, including host colonization and antimicrobial resistance. Activation of PNAG production in *S. aureus* not only contributed to biofilm formation on the surface of implanted catheters [71] but also slowed down neutrophil recruitment and bacterial clearance in mice challenged intraperitoneally with *S. epidermidis* biofilm cells of a PNAG-defective mutant [72]. Biofilm-producing strains of *P. aeruginosa* are a major cause of morbidity and mortality in cystic fibrosis (CF) patients. In an animal model, increased levels of Th2 cytokines and macrophages were primarily EPS-driven rather than by the pathogen *P. aeruginosa* itself [73].

The secretion of proteases and other extracellular enzymes, such as lipase, esterase, DNase, RNase, and fibrinolysin, is an essential process for bacterial growth and virulence. For instance, bacterial glycosyltransferases and their EPS glucan products have been extensively studied for their functional roles in the pathogenesis of dental caries (tooth decay) in *in vivo* and clinical studies [5,74]. Glucans promote bacterial adhesion–cohesion, interspecies interactions, and biofilm accumulation as well as helping to create protective and highly acidic microenvironments [5,74]. Recently, a putative glucosyltransferase in *S. mutans* (SMU_833) was found to modulate dynamic interactions between two key biofilm matrix components, glucan and eDNA. The deletion of *smu_833* decreases glucan and increases eDNA but maintains the overall biofilm biomass with reduced virulence in an *in vivo* rat model of dental caries [75]. Dextranase (Dex) is a type of glucanase of *S. mutans*, participating in the modification and degradation of extracellular water-soluble glucans, which has been associated with caries development in rats [76].

Bacterial pathogens, such as *P. aeruginosa* and *S. aureus*, have developed a complex network of evasion, counter-inhibition, and subjugation in their battle for space and nutrients. Notably, EPS components also play an important role for interspecies interactions between these organisms *in vivo* [25]. The assembly of enteric biofilm is essential to understand the role of EPS virulence. The EPS of enteric biofilms – mainly including amyloid curli, eDNA, O antigen, cellulose, and surface proteins such as BapA – are etiologically associated with the autoimmune disease lupus erythematosus (SLE) and can activate the fibrillization of the Parkinson's disease-related amyloid α-synuclein [77]. Enteric EPS components have long been recognized as pathogen-associated molecular patterns (PAMPs) that can activate proinflammatory innate immune receptors, such as Toll-like receptors (TLR2, TLR9), CD14 heterocomplex, and NLRP3. Curli are extracellular amyloid fibers produced by enterobacteria such as *E. coli* and *Salmonella*. These fibers have been shown to play an important role in biofilm formation as they display direct interaction with the substratum and form bundles between bacterial cells, thus allowing a cohesive and stable association of cells in the biofilm. Curli have been found to not only protect *E. coli* but to enhance its virulence potential by blocking the actions of C1q [34,78,79]. Other protein fibers, often described as functional amyloid or amyloid-like fibers – for example the Fap fibers in *Pseudomonas* sp., the nonamyloidogenic TasA fibers formed by *B. subtilis*, and the Bap fibers of *S. aureus* – are also commonly present in bacterial biofilms. Their structural/functional importance and link to biofilm virulence has been reviewed extensively elsewhere [34]. However, it is important to note that there is great diversity in composition of the amyloid fibers formed by a broad range of species; this needs further elucidation, especially in how they contribute to the biofilm virulence collectively in a polymicrobial community.

In *C. albicans*, the most common opportunistic fungal pathogen, multiple matrix components (e.g., extracellular polysaccharides such as β-glucans and mannan) have been associated with virulence. These matrix components can protect *C. albicans* from antifungal drugs and host immune systems [18,80]. Using an *in vivo* venous catheter biofilm model, *C. albicans* biofilms were shown to impair neutrophil response through an inhibitory pathway induced by EPS [81]. Moreover, *Candida* spp. are frequently implicated in mixed bacterial-fungal infections forming biofilms that cause many infections in humans, particularly mucosal diseases [82]. *Candida*-derived β-glucans and extracellular enzymes such as phospholipases, lipases, and hemolysins have been associated with infections of the bloodstream, implant, vagina, and oral cavity [83]. *C. albicans* virulence has also been associated with eDNA and extracellular vesicles carrying several virulence factors that were found to stimulate immune responses in macrophages and dendritic cells [84]. In addition to mucosal infections, *C. albicans* also appears to interact with *S. mutans* on tooth surfaces, forming mixed-kingdom biofilms associated with severe early childhood caries. Such interactions modify the biofilm environment by boosting the amounts of both bacterial and fungal-derived EPS, increasing the biofilm mass, density of infection, and acidogenicity, leading to enhanced caries severity *in vivo* [31,45,46].

Combating the Matrixome via Multitargeted Strategies

The multifaceted nature of biofilm matrixome imposes great challenges to combat biofilm-related diseases. Due to the complex physical and biological properties of EPS matrix, biofilm infections are

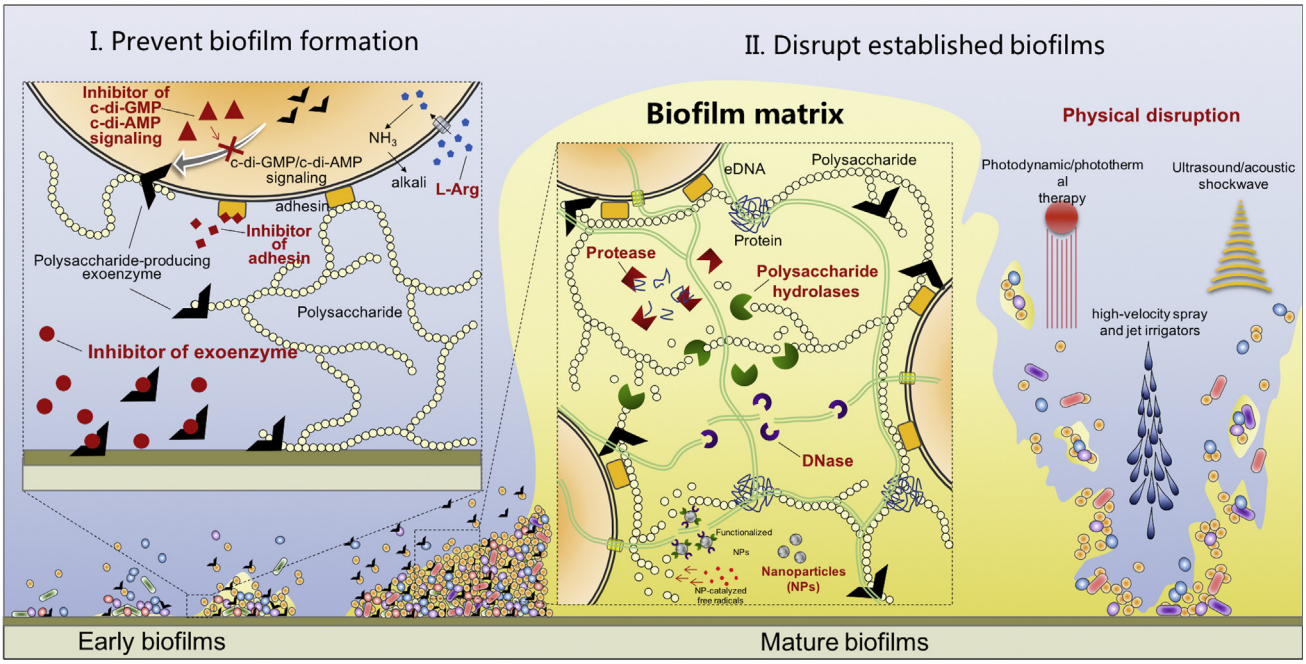

Figure 2. Therapeutic Strategies Targeting the Biofilm Matrix. The matrix is a multifunctional scaffold that is essential for the entire biofilm's life cycle, from initial microbial colonization to biofilm maturation, and expression of virulence. Biofilms are not amenable to conventional antimicrobial approaches due to the complex physical and biological properties of the matrix. Targeting the matrix, especially the extracellular polymeric substances (EPS) elements that are universal among different species (e.g., exopolysaccharides, proteins, and eDNA) may have the potential to achieve cross-species antibiofilm efficacy in a polymicrobial infection. Therapeutic strategies can be designed to prevent the biofilm formation either by inhibiting EPS production or blocking adhesin-mediated adherence (left). When biofilms are already established, strategies that can degrade EPS macromolecules may dismantle the scaffolding/protective matrix to weaken the biofilm structure and potentiate antimicrobial killing (middle). In cases where biofilm removal is favorable, and aggressive measures are needed (e.g., dental hygiene and surgical debridement of infection sites), the EPS network can also be disrupted using physical-mechanical methods.

often not amenable to conventional antimicrobial approaches, requiring multitargeted or combinatorial therapies. Current and prospective therapeutic strategies targeting the vital structural and functional traits in EPS have been recently reviewed [4,85]. However, knowledge about EPS-targeting approaches has been derived mostly from single-species biofilms, and studies using polymicrobial systems remain sparse.

In general, targeting can be achieved by inhibiting the production of EPS, blocking adhesin-mediated adherence and/or by degrading EPS in established biofilms (Figure 2; see also Table S1 in the supplemental information online). Biofilm EPS production is regulated by several extracellular/intracellular signaling networks and nonsignaling mechanisms, which can be targeted to control biofilm formation [2,3,66,86]. Blocking EPS-mediated adherence via inhibitors of adhesin production or adhesin-binding molecules targets EPS–host interaction to prevent bacterial and fungal biofilm initiation. However, many microorganisms exhibit multiple receptor–ligand interactions that allow binding to host surfaces. Therefore, more than a single adhesive process must be impeded for the bacterial colonization to be blocked, which requires better knowledge of the biology and stereochemistry of adhesins [60]. The extracellular nucleoproteins (DNABII family) that provide structural integrity to eDNA were also targeted using antibodies, resulting in destabilization of the biofilm structure. Targeting DNABII showed *in vivo* efficacy in various biofilms found in oral, urinary tract, and pulmonary infections, indicating its therapeutic potential in polymicrobial infections [87–89]. Interestingly, antiamyloid drugs originally designed to target human pathological amyloids (e.g., in Alzheimer's and Parkinson's diseases) were repurposed as novel antibiofilm agents, inspired by the recent finding of the conserved fibrillar architecture between bacterial and human amyloids [79]. When biofilms pre-exist with a substantial amount of matrix already formed, strategies that inhibit EPS synthesis or block the adhesion interactions may not be successful due to the presence of established and protective microenvironments [4]. In this case, it may be necessary to degrade the formed EPS using enzymes and other agents to disrupt the physical integrity of the matrix, and facilitate biofilm disruption and removal [4]. For maximal efficacy, EPS-degrading enzymes can be used as adjunctive agents for conventional antimicrobials that enhance drug penetrability and microbial killing activity, while promoting breakdown of the biofilm structure [69,90]. In addition, physical removal, including mechanical, energy-based and light-based disruption, may further improve EPS-based intervention strategies [4].

New technologies and bio/nanomaterials could provide alternative ways to control EPS-mediated biofilm virulence. For example, on-demand 'smart release' nanocarriers that can penetrate biofilms and be triggered by pathogenic microenvironments to deliver drugs or multifunctional compounds (from catalytic nanoparticles to aptamers, dendrimers, and bioactive peptides) have been developed to disrupt the EPS and the viability or metabolic activity of the embedded bacteria. The use of probiotics or phage therapy targeting EPS has also been considered as potential antibiofilm strategies. Nevertheless, questions remain on the efficacy of solely targeting EPS as it may not necessarily address bacterial viability. Given the complexity of biofilm biology, therapeutic strategies that target both the microorganisms and the EPS matrix – to either prevent biofilm initiation or disrupt existing biofilms – appear to be a more effective and precise approach.

Outstanding Questions

How does the dynamic EPS synthesis and degradation (remodeling) influence the physicochemical properties of the matrix, microbial signaling, and communal behavior within the biofilm?

What are the roles of nucleic acids (eDNA, ribosomal DNA, and eRNA) and eDNA–protein or eDNA–polysaccharide complexes found in the biofilm matrix?

How does the bacterial phenotypic and genomic heterogeneity affect EPS matrix assembly and biofilm development?

How do the physicochemical properties of the matrix modulate the development of microenvironments and pathogenic niches in polymicrobial biofilm communities?

How does the microenvironment heterogeneity impact interspecies or interkingdom interactions and virulence of polymicrobial biofilms?

Future Directions and Perspectives

The biofilm matrix is more than a scaffold to maintain the biofilm structure. EPS participates actively in functional properties that are essential for biofilm assembly, persistence, collective behavior, and virulence, such as signaling, genetic exchange, creation of microenvironments, mechanical stability, and antimicrobial tolerance. Moreover, matrix is being constantly formed and remodeled, affecting structure and function throughout the biofilm's life cycle.

It is noteworthy that biofilms constantly restructure, and the microenvironment dynamically changes, whereas most of the data available have been gathered in observations taken at individual points in time or in static conditions. Therefore, further investigations should focus on the dynamics of regulation of matrix formation, structural organization, and remodeling, which may lead to new insights into the composition and structure of EPS in biofilms. These can be facilitated using recent technological advances in time-lapse super-resolution imaging and *in situ* spectroscopy-based methods combined with computational analyses. Furthermore, real-time metabolite profiling combined with new labeling methods using pH/O_2-sensitive fluorescent probes cross-linked to different EPS components may reveal the spatiotemporal dynamics of microenvironments developing at different locations throughout the biofilm architecture.

However, the presence of different species, and even different kingdoms, further complicates compositional analyses and biological properties of EPS. While more details of the EPS matrix composition in polymicrobial biofilms are being revealed, the knowledge about its functional role and structural organization – as well as mechanistic details on how different microbes collectively regulate production and interact with the EPS components within intact biofilms – remains limited. We emphasize the need for the development of robust and reproducible polymicrobial biofilms using laboratory and *in vivo* models to help assess the functional role of the various types of EPS from different organisms in a mixed community.

Enhanced understanding of the multifaceted nature of biofilm matrix can also lead to more efficacious approaches to control biofilm-related diseases. Variability of the EPS composition and structure – depending on the microbial species and metabolic activity, nutrient availability, host environment, and growth stage – poses significant hurdles to the development of EPS-targeting therapeutics. Conventional approaches focused solely on **EPS degradation** (or antimicrobial activity) may not achieve efficacy within the complex (physicochemical) biofilm microenvironment. Prospective therapeutic strategies need to target simultaneously the biofilm matrix components and the embedded microorganisms to eradicate the pathogenic niche with minimal cytotoxicity to surrounding tissues. A potential way to enhance precision and efficacy may involve 'stimuli-triggered bioactivity/drug delivery' or 'on-demand activation' in response to biofilm-specific conditions, including EPS production, bacterial activity, and chemical environment (such as pH and O_2) in addition to enhanced drug penetration. However, rigorous assessment of efficacy, feasibility, and biocompatibility of novel approaches is required using clinically relevant *in vivo* models, which remain underdeveloped. Future studies addressing current limitations may lead to breakthroughs in biofilm basic research and clinical translation of prospective therapeutics (see Outstanding Questions).

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References

1. Flemming, H.C. *et al.* (2016) Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* 14, 563–575
2. Koo, H. and Yamada, K.M. (2016) Dynamic cell-matrix interactions modulate microbial biofilm and tissue 3D microenvironments. *Curr. Opin. Cell Biol.* 42, 102–112
3. Dragos, A. and Kovacs, A.T. (2017) The peculiar functions of the bacterial extracellular matrix. *Trends Microbiol.* 25, 257–266
4. Koo, H. *et al.* (2017) Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat. Rev. Microbiol.* 15, 740–755
5. Bowen, W.H. *et al.* (2018) Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol.* 26, 229–242
6. Marsh, P.D. *et al.* (2016) Influence of saliva on the oral microbiota. *Periodontology* 2000, 80–92

7. Stickler, D. *et al.* (1998) Studies on the formation of crystalline bacterial biofilms on urethral catheters. *Eur. J. Clin. Microbiol. Infect. Dis.* 17, 649–652
8. Keren-Paz, A. *et al.* (2018) Micro-CT X-ray imaging exposes structured diffusion barriers within biofilms. *NPJ Biofilms Microbiomes* 4, 8
9. Oppenheimer-Shaanan, Y. *et al.* (2016) Spatio-temporal assembly of functional mineral scaffolds within microbial biofilms. *NPJ Biofilms Microbiomes* 2, 15031
10. Dade-Robertson, M. *et al.* (2017) Architects of nature: growing buildings with bacterial biofilms. *Microb. Biotechnol.* 10, 1157–1163
11. Li, X. *et al.* (2015) Spatial patterns of carbonate biomineralization in biofilms. *Appl. Environ. Microbiol.* 81, 7403–7410
12. van Gestel, J. *et al.* (2015) From cell differentiation to cell collectives: *Bacillus subtilis* uses division of labor to migrate. *PLoS Biol.* 13, e1002141
13. Angelini, T.E. *et al.* (2009) *Bacillus subtilis* spreads by surfing on waves of surfactant. *Proc. Natl. Acad. Sci. U. S. A.* 106, 18109–18113
14. Grau, R.R. *et al.* (2015) A duo of potassium-responsive histidine kinases govern the multicellular destiny of *Bacillus subtilis*. *mBio* 6, e00581
15. Ogran, A. *et al.* (2019) The plant host induces antibiotic production to select the most-beneficial colonizers. *Appl. Environ. Microbiol.* 85, e00512–e00519
16. Hobley, L. *et al.* (2015) Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol. Rev.* 39, 649–669
17. Peterson, B.W. *et al.* (2015) Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiol. Rev.* 39, 234–245
18. Zarnowski, R. *et al.* (2014) Novel entries in a fungal biofilm matrix encyclopedia. *mBio* 5, e01333-14
19. Teschler, J.K. *et al.* (2015) Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nat. Rev. Microbiol.* 13, 255–268
20. Vlamakis, H. *et al.* (2013) Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.* 11, 157–168
21. Jones, C.J. and Wozniak, D.J. (2017) Psl produced by mucoid *Pseudomonas aeruginosa* contributes to the establishment of biofilms and immune evasion. *mBio* 8, e00864-17
22. Harimawan, A. and Ting, Y.P. (2016) Investigation of extracellular polymeric substances (EPS) properties of *P. aeruginosa* and *B. subtilis* and their role in bacterial adhesion. *Colloids Surf. B Biointerf.* 146, 459–467
23. Toska, J. *et al.* (2018) Exopolysaccharide protects *Vibrio cholerae* from exogenous attacks by the type 6 secretion system. *Proc. Natl. Acad. Sci. U. S. A.* 115, 7997–8002
24. Yan, J. *et al.* (2016) *Vibrio cholerae* biofilm growth program and architecture revealed by single-cell live imaging. *Proc. Natl. Acad. Sci. U. S. A.* 113, E5337–E5343
25. Hotterbeekx, A. *et al.* (2017) *In vivo* and *in vitro* interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Front. Cell. Infect. Microbiol.* 7, 106
26. Xiao, J. *et al.* (2012) The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathog.* 8, e1002623
27. Berk, V. *et al.* (2012) Molecular architecture and assembly principles of *Vibrio cholerae* biofilms. *Science* 337, 236–239
28. Drescher, K. *et al.* (2016) Architectural transitions in *Vibrio cholerae* biofilms at single-cell resolution. *Proc. Natl. Acad. Sci. U. S. A.* 113, E2066–E2072
29. Wang, C. *et al.* (2019) Emergent properties in *Streptococcus mutans* biofilms are controlled through adhesion force sensing by initial colonizers. *mBio* 10, e01908-19
30. Falsetta, M.L. *et al.* (2014) Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms *in vivo*. *Infect. Immun.* 82, 1968–1981
31. Hwang, G. *et al.* (2017) *Candida albicans* mannans mediate *Streptococcus mutans* exoenzyme GtfB binding to modulate cross-kingdom biofilm development *in vivo*. *PLoS Pathog.* 13, e1006407
32. Duanis-Assaf, D. *et al.* (2018) Cell wall associated protein TasA provides an initial binding component to extracellular polysaccharides in dual-species biofilm. *Sci. Rep.* 8, 9350
33. Jayathilake, P.G. *et al.* (2017) Extracellular polymeric substance production and aggregated bacteria colonization influence the competition of microbes in biofilms. *Front. Microbiol.* 8, 1865
34. Erskine, E. *et al.* (2018) Functional amyloid and other protein fibers in the biofilm matrix. *J. Mol. Biol.* 430, 3642–3656
35. Sullan, R.M. *et al.* (2015) Binding forces of *Streptococcus mutans* P1 adhesin. *ACS Nano* 9, 1448–1460
36. Romero, D. *et al.* (2010) Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2230–2234
37. Dueholm, M.S. *et al.* (2010) Functional amyloid in *Pseudomonas*. *Mol. Microbiol.* 77, 1009–1020
38. Taglialegna, A. *et al.* (2016) Staphylococcal Bap proteins build amyloid scaffold biofilm matrices in response to environmental signals. *PLoS Pathog.* 12, e1005711
39. Turnbull, L. *et al.* (2016) Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* 7, 11220
40. Reichhardt, C. and Parsek, M.R. (2019) Confocal laser scanning microscopy for analysis of *Pseudomonas aeruginosa* biofilm architecture and matrix localization. *Front. Microbiol.* 10, 677
41. Hou, J. *et al.* (2018) Extracellular polymeric matrix production and relaxation under fluid shear and mechanical pressure in *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol.* 84, e01516–e01517
42. Karygianni, L. *et al.* (2014) Novel broad-spectrum antimicrobial photoinactivation of *in situ* oral biofilms by visible light plus water-filtered infrared A. *Appl. Environ. Microbiol.* 80, 7324–7336
43. Hall, C.W. and Mah, T.F. (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol. Rev.* 41, 276–301
44. Kong, E.F. *et al.* (2016) Commensal protection of *Staphylococcus aureus* against antimicrobials by *Candida albicans* biofilm matrix. *mBio* 7, e01365-16
45. Kean, R. *et al.* (2017) *Candida albicans* mycofilms support *Staphylococcus aureus* colonization and enhances miconazole resistance in dual-species interactions. *Front. Microbiol.* 8, 258
46. Kim, D. *et al.* (2018) Bacterial-derived exopolysaccharides enhance antifungal drug tolerance in a cross-kingdom oral biofilm. *ISME J.* 12, 1427–1442
47. Townsend, E.M. *et al.* (2017) Implications of antimicrobial combinations in complex wound biofilms containing fungi. *Antimicrob. Agents Chemother.* 61, e00672-17
48. Townsend, E.M. *et al.* (2016) Development and characterisation of a novel three-dimensional inter-kingdom wound biofilm model. *Biofouling* 32, 1259–1270
49. Arnaouteli, S. *et al.* (2017) Bifunctionality of a biofilm matrix protein controlled by redox state. *Proc. Natl. Acad. Sci. U. S. A.* 114, E6184–E6191
50. Morris, R.J. *et al.* (2017) Natural variations in the biofilm-associated protein BslA from the genus *Bacillus*. *Sci. Rep.* 7, 6730
51. Yahav, S. *et al.* (2018) Encapsulation of beneficial probiotic bacteria in extracellular matrix from biofilm-forming *Bacillus subtilis*. *Artif. Cells Nanomed. Biotechnol.* 46, 974–982
52. Nadell, C.D. *et al.* (2017) Flow environment and matrix structure interact to determine spatial competition in *Pseudomonas aeruginosa* biofilms. *eLife* 6, e21855
53. Lawrence, J.R. *et al.* (2016) *In situ* evidence for metabolic and chemical microdomains in the structured polymer matrix of bacterial microcolonies. *FEMS Microbiol. Ecol.* 92, fiw183
54. Sonderholm, M. *et al.* (2018) Tools for studying growth patterns and chemical dynamics of aggregated *Pseudomonas aeruginosa* exposed to different electron acceptors in an alginate bead model. *NPJ Biofilms Microbiomes* 4, 3
55. Beebout, C.J. *et al.* (2019) Respiratory heterogeneity shapes biofilm formation and host colonization in uropathogenic *Escherichia coli*. *mBio* 10, e02400-18
56. Hwang, G. *et al.* (2016) Simultaneous spatiotemporal mapping of *in situ* pH and bacterial activity within an intact 3D microcolony structure. *Sci. Rep.* 6, 32841

57. Schlafer, S. *et al.* (2018) Improved pH-ratiometry for the three-dimensional mapping of pH microenvironments in biofilms under flow conditions. *J. Microbiol. Methods* 152, 194–200
58. Guo, L. *et al.* (2015) The well-coordinated linkage between acidogenicity and aciduricity via insoluble glucans on the surface of *Streptococcus mutans*. *Sci. Rep.* 5, 18015
59. Cugini, C. *et al.* (2019) The role of exopolysaccharides in oral biofilms. *J. Dent. Res.* 98, 739–745
60. Yan, J. *et al.* (2017) Extracellular-matrix-mediated osmotic pressure drives *Vibrio cholerae* biofilm expansion and cheater exclusion. *Nat. Commun.* 8, 327
61. Bertolini, M.M. *et al.* (2015) *Candida*-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotypes. *Mol Oral Microbiol* 30, 307–322
62. DeFrancesco, A.S. *et al.* (2017) Genome-wide screen for genes involved in eDNA release during biofilm formation by *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U. S. A.* 114, E5969–E5978
63. Okshevsky, M. and Meyer, R.L. (2015) The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. *Crit. Rev. Microbiol.* 41, 341–352
64. LewisOscar, F. *et al.* (2018) *In vitro* and *in silico* attenuation of quorum sensing mediated pathogenicity in *Pseudomonas aeruginosa* using *Spirulina platensis*. *Microb. Pathog.* 116, 246–256
65. Whiteley, M. *et al.* (2017) Progress in and promise of bacterial quorum sensing research. *Nature* 551, 313–320
66. Steinberg, N. and Kolodkin-Gal, I. (2015) The matrix reloaded: probing the extracellular matrix synchronizes bacterial communities. *J. Bacteriol.* 197, 2092–2103
67. Kim, M.K. *et al.* (2017) Surface-attached molecules control *Staphylococcus aureus* quorum sensing and biofilm development. *Nat. Microbiol.* 2, 17080
68. Bai, Y. *et al.* (2017) Crystal structure of 4,6-alpha-glucanotransferase supports diet-driven evolution of GH70 enzymes from alpha-amylases in oral bacteria. *Structure* 25, 231–242
69. Ren, Z. *et al.* (2019) Dual-targeting approach degrades biofilm matrix and enhances bacterial killing. *J. Dent. Res.* 98, 322–330
70. Giaouris, E. *et al.* (2015) Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. *Front. Microbiol.* 6, 841
71. Burgui, S. *et al.* (2018) A systematic evaluation of the two-component systems network reveals that arlrs is a key regulator of catheter colonization by *Staphylococcus aureus*. *Front. Microbiol.* 9, 342
72. Ferreirinha, P. *et al.* (2016) Poly-*N*-acetylglucosamine production by *Staphylococcus epidermidis* cells increases their *in vivo* proinflammatory effect. *Infect. Immun.* 84, 2933–2943
73. Bielen, K. *et al.* (2017) Biofilm-induced type 2 innate immunity in a cystic fibrosis model of *Pseudomonas aeruginosa*. *Front. Cell. Infect. Microbiol.* 7, 274
74. Zhu, F. *et al.* (2015) Glycosyltransferase-mediated sweet modification in oral streptococci. *J. Dent. Res.* 94, 659–665
75. Rainey, K. *et al.* (2019) Glycosyltransferase-mediated biofilm matrix dynamics and virulence of *Streptococcus mutans*. *Appl. Environ. Microbiol.* 85, e02247-18
76. Yang, Y. *et al.* (2019) Regulation of water-soluble glucan synthesis by the *Streptococcus mutans* dexA gene effects biofilm aggregation and cariogenic pathogenicity. *Mol Oral Microbiol* 34, 51–63
77. Tursi, S.A. and Tukel, C. (2018) Curli-containing enteric biofilms inside and out: matrix composition, immune recognition, and disease implications. *Microbiol. Mol. Biol. Rev.* 82, e00028-18
78. Biesecker, S.G. *et al.* (2018) The functional amyloid curli protects *Escherichia coli* against complement-mediated bactericidal activity. *Biomolecules* 8, E5
79. Perov, S. *et al.* (2019) Structural insights into Curli CsgA cross-beta fibril architecture inspire repurposing of anti-amyloid compounds as anti-biofilm agents. *PLoS Pathog.* 15, e1007978
80. Gunn, J.S. *et al.* (2016) What's on the outside matters: the role of the extracellular polymeric substance of gram-negative biofilms in evading host immunity and as a target for therapeutic intervention. *J. Biol. Chem.* 291, 12538–12546
81. Johnson, C.J. *et al.* (2016) The extracellular matrix of *Candida albicans* biofilms impairs formation of neutrophil extracellular traps. *PLoS Pathog.* 12, e1005884
82. Allison, D.L. *et al.* (2016) *Candida*-bacteria interactions: their impact on human disease. *Microbiol. Spectr.* Published online May 13, 2016. https://doi.org/10.1128/microbiolspec.VMBF-0030-2016
83. Rodrigues, C.F. *et al.* (2017) *Candida glabrata* biofilms: how far have we come? *J. Fungi (Basel)* 3, 11
84. Hirota, K. *et al.* (2017) Pathogenic factors in *Candida* biofilm-related infectious diseases. *J. Appl. Microbiol.* 122, 321–330
85. Liu, Y. *et al.* (2018) Therapeutic strategies targeting cariogenic biofilm microenvironment. *Adv. Dent. Res.* 29, 86–92
86. Pérez-Mendoza, D. and Sanjuán, J. (2016) Exploiting the commons: cyclic diguanylate regulation of bacterial exopolysaccharide production. *Curr. Opin. Microbiol.* 30, 36–43
87. Rocco, C.J. *et al.* (2017) Natural antigenic differences in the functionally equivalent extracellular DNABII proteins of bacterial biofilms provide a means for targeted biofilm therapeutics. *Mol Oral Microbiol* 32, 118–130
88. Devaraj, A. *et al.* (2015) DNABII proteins play a central role in UPEC biofilm structure. *Mol. Microbiol.* 96, 1119–1135
89. Novotny, L.A. *et al.* (2016) Monoclonal antibodies against DNA-binding tips of DNABII proteins disrupt biofilms *in vitro* and induce bacterial clearance *in vivo*. *EBioMedicine* 10, 33–44
90. Snarr, B.D. *et al.* (2017) Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity. *Proc. Natl. Acad. Sci. U. S. A.* 114, 7124–7129
91. Theilade, J. (1977) Development of bacterial plaque in the oral cavity. *J. Clin. Periodontol.* 4, 1–12
92. Huynh, H.T. *et al.* (2016) Bacteria and archaea paleomicrobiology of the dental calculus: a review. *Mol. Oral Microbiol.* 31, 234–242
93. Guggenheim, B. (1970) Extracellular polysaccharides and microbial plaque. *Int. Dent. J.* 20, 657–678
94. Bowen, W.H. and Guggenheim, B. (1978) Therapeutics of caries prevention – concepts and prospects. *Acta Odontol. Scand.* 36, 185–198
95. Thurnheer, T. *et al.* (2016) Microbial dynamics during conversion from supragingival to subgingival biofilms in an *in vitro* model. *Mol Oral Microbiol* 31, 125–135
96. Schlafer, S. and Meyer, R.L. (2017) Confocal microscopy imaging of the biofilm matrix. *J. Microbiol. Methods* 138, 50–59
97. Mann, E.E. and Wozniak, D.J. (2012) *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiol. Rev.* 36, 893–916
98. Nett, J.E. and Andes, D.R. (2020) Contributions of the biofilm matrix to *Candida* pathogenesis. *J. Fungi (Basel)* 6, 21
99. Ibáñez de Aldecoa, A.L. *et al.* (2017) Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. *Front. Microbiol.* 8, 1390
100. Cochet, F. and Peri, F. (2017) The role of carbohydrates in the lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signalling. *Int. J. Mol. Sci.* 18, 2318